Plant Physiol. (1991) 96, 1150–1156
0032-0889/91/96/1150/07/$01.00/0

Received for publication October 3, 1990
Accepted March 5, 1991

Occurrence of an Inhibitor of Tissue-Type Plasminogen Activator in Seeds and *in Vitro* Cultures of *Erythrina caffra* Thunb[1]

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ABSTRACT

The level of an inhibitor of tissue-type plasminogen activator (t-PA) increased slowly during the early developmental stage of seeds of *Erythrina caffra* Thunb. Thereafter, the inhibitor increased exponentially until the seeds reached maturity. At maturity, the t-PA inhibitor levels in the cotyledons were 38 times higher than the levels at the onset of seed development. The t-PA inhibitor accumulated at a faster rate than the storage proteins, which reached a concentration 15 times higher than the protein concentration at the onset of seed development. During the imbibition and germination process, the t-PA inhibitor decreased gradually. The inhibitor kept on decreasing during the growth of the seedlings until the 10th day after imbibition, when it leveled off at 4.1% of that of the initial inhibitor concentration. The inhibitor remained at this level until the cotyledons were shed at day 22. The total protein in the cotyledons decreased at a slower rate than the inhibitor and reached a minimum concentration at day 20 of 3.6% of the initial protein concentration in the cotyledons. Callus cultures of root, shoot, leaf, and cotyledonary tissue was established and maintained on Murashige-Skoog medium supplemented with 3% sucrose, 10 micromolar benzyladenine, and 5 micromolar 2,4-dichlorophenoxyacetic acid. A shoot cell suspension culture was established on Murashige-Skoog medium supplemented with 3% sucrose, 1 micromolar benzyladenine, and 0.5 micromolar 2,4-dichlorophenoxyacetic acid (pH 5.7) and shaken at 60 revolutions per minute. The level of t-PA inhibitor in root, shoot, leaf, and cotyledonary callus was substantially lower than in the corresponding intact tissue. The t-PA inhibitor levels in the linear growth phase was higher than in the lag or stationary growth phases of the cell suspension culture. A hydrolysate of the cell walls of tomato and *E. caffra* Thunb, as well as polyamines and organic acids, did not increase the concentration of t-PA inhibitor in suspension cultures or intact leaf tissue of *E. caffra*. The t-PA inhibitor levels of suspension cultures were increased by Na_2SO_4 but not by L-cysteine in the nutrient medium.

Plant proteins that are inhibitory toward enzymes occurring in animals have been studied extensively (25). The Kunitz-type trypsin inhibitors comprise one group of these inhibitors. The majority of trypsin inhibitors isolated from plants are specific for either trypsin or chymotrypsin, with weak or no activity for the second enzyme. Inhibitors originally isolated as Kunitz-type trypsin inhibitors from *Erythrina caffra* Thunb. (14) were reported to inhibit the fibrinolytic serine proteinase t-PA[2] (16). Apart from its function in normal tissue, t-PA is secreted in large quantities by oncogenic cells (7). These t-PA inhibitors isolated from seeds of *E. caffra* and other members of the genus *Erythrina* had a substantially higher affinity for t-PA than trypsin and chymotrypsin (16). The t-PA inhibitors were recently classified as a new class of inhibitor in the family of trypsin inhibitors (16). Kunitz-type trypsin inhibitors occur mainly in storage organs such as seeds and their levels in seeds are affected by the germination process. It has been reported that proteinase inhibitors in members of the Solanaceae and carrot occur *in vitro* (5, 27). Proteinase inhibitors can be induced to accumulate *in vitro* in members of the Solanaceae (27). It has not yet been established whether the t-PA inhibitor occurs in larger quantities in other parts of the plant and whether the amount of inhibitor in seeds is affected by seed development or germination.

We report here on the distribution of t-PA inhibitor in *in vivo* and *in vitro* cultures of *E. caffra* and on the effect of seed development and germination on the concentration of the inhibitor in cotyledonary tissue.

MATERIALS AND METHODS

To determine the t-PA inhibitor content of *Erythrina caffra* seeds during the course of their development, seeds of different sizes were collected. The seeds were removed from the pods at the day of collection and classified according to their length to serve as a measure of development. Thereafter, the seeds were frozen with liquid nitrogen and stored at −20°C until further use. The experiment was repeated three times over a 3 year period with seeds from the same tree.

To germinate seeds under aseptic conditions, lots of 50 seeds were surface-sterilized for 20 min with 0.2% $HgCl_2$ containing 0.01% (v/v) Tween 20. Thereafter, they were rinsed with 5 × 200 mL autoclaved, distilled water. The seeds were scarified with concentrated H_2SO_4 for 30 min at 25°C and then rinsed with 5 × 200 mL autoclaved, distilled water. Thereafter, they were imbibed overnight and incubated under aseptic conditions on moist Whatman No. 1 filter paper in

[1] Supported by the Foundation for Research Development and Technifin.

[2] Abbreviation: t-PA, tissue-type plasminogen activator.

glass jars at 27°C in the light (17). Three seeds were removed every 2 h during the first 48 h of incubation. Thereafter, seeds were removed every second day until the cotyledons were shed. The fresh mass of the cotyledons was determined and they were then frozen with liquid nitrogen and stored at −20°C until further use. Germination was considered to have occurred when the radicle became visible. Subsequent development was defined as seedling growth.

Callus Culture

Shoot and leaf material used for *in vitro* cultures was obtained from greenhouse-grown *E. caffra* donor plants (17). The shoot and leaf explant material obtained from the donor plants was surface-sterilized with 0.2% $HgCl_2$ and 0.01% Tween 20. The plant material was then rinsed in 5 × 100 mL autoclaved, distilled water. Root material was obtained from seedlings grown aseptically as described before. Cotyledonary and embryonic tissue was obtained from seeds imbibed under aseptic conditions as described before. Callus cultures of root, shoot, leaf, cotyledonary, and embryonic tissue were initiated and grown on a basal medium consisting of the salts, vitamins, and mesoinositol of Murashige and Skoog (17) supplemented with 3% sucrose, 10 μM benzyladenine, and 5 μM 2,4-D. The pH was adjusted to 5.7. The medium was autoclaved for 20 min at 121°C and solidified with 0.2% Gelrite. With the experiments conducted, the callus was harvested, the excess fluid absorbed on paper toweling, and the fresh mass determined. Thereafter, the callus was frozen in liquid nitrogen and stored at −20°C.

Cell Suspension Cultures

Suspension cultures of shoot-derived callus were established with friable callus on the basal medium supplemented with 1 μM BA and 0.5 μM 2,4-D. Experimental cultures with a volume of 30 mL in 100 mL Erlenmeyer flasks were shaken at 60 rpm on an orbital shaker with an orbital radius of 1.5 cm. The cultures were incubated at 25°C under a quantum flux density (400–700 nm) of 5 to 10 μmol m^{-2} s^{-1} produced by fluorescent tubes. The growth of the cell suspension cultures was recorded by determining the settled cell volume (17). The viability of the cell suspension cultures was determined with fluorescein diacetate (17). Cell suspensions in the exponential growth phase were used for the experiments. The cells were washed with 3 × 10 mL sterile treatment solution before they were incubated. Treated cells harvested from the suspension cultures were rinsed with 3 × 10 mL of distilled water under vacuum filtration. Thereafter, the fresh mass was determined. The cells were immediately frozen in liquid nitrogen and stored at −20°C. The growth index was determined by dividing the increase in settled cell volume by the initial value.

Several protein-inducing substances were used in an effort to increase the levels of t-PA inhibitor in the cell suspension cultures. The protein-inducing substances added to the basal nutrient medium were spermine (0.1 mM), spermidine (0.1 mM), benzoic acid (0.1 mM), phytic acid (0.1 mM), salicylic acid (0.1 mM), and ethylene glycol chitin (1 mg L^{-1}). These compounds were dissolved in basal medium and added to the cell suspension medium. Cell wall hydrolysate of *E. caffra* and *Lycopersicon esculentum* Mill. were added to the nutrient medium to obtain a final concentration of 0.1 mg L^{-1}. The cell wall hydrolysate was prepared according to a method modified from Hahn *et al.* (17). The cells were harvested after an incubation period of 48 h.

Two experiments were conducted to determine the effect of organic and inorganic sources of S^{-2} on the t-PA inhibitor content of cell suspension cultures. In the first experiment, the S^{-2} concentration in the basal medium was adjusted to 0.173, 0.346, 1.73, 8.65, and 17.3 mM, respectively. This corresponds to 0.1, 0.2, 1, 5, and 10 times the concentration of S^{-2} in the original basal medium. The S^{-2} levels in the basal medium was regulated by decreasing the original $MgSO_4$ concentration or by supplementing the nutrient media with Na_2SO_4. The concentration of magnesium was kept constant at 1.73 mM with $MgCO_3$. In the second experiment, the treatments consisted of 0.173, 1.73, and 8.65 mM of S^{-2} with and without 0.1 mM of L-cysteine in the basal medium. Volumes of 100 mL cell suspension in treatment solution were incubated in 500 mL Erlenmeyer flasks under the standard incubation conditions. A volume of 20 mL of each treated cell suspension was harvested after 7 d incubation. The rest of the cultures were maintained to determine the effect of S^{-2} and cysteine on growth.

Lipids and Chl were extracted with acetone and ethyl ether from cotyledons and green plant tissues, respectively, and the acetone dry mass was determined (17). Protein was extracted from the plant tissue with a buffered saline solution (pH 8.0) containing 500 mM NaCl, 100 mM Tris-HCL, and 40 mM mercaptoethanol (17). The protein extract was fractionated with 60% (w/v) ammonium sulfate and dialyzed overnight at 10°C. Thereafter, the protein was lyophilized and stored in a desiccator. Protein was quantified with the dye-binding assay of Read and Northcote (17). The t-PA inhibitor content (μg·g^{-1}) of the 60% ammonium sulfate protein fractions was determined with a double sandwich ELISA (17). The experiments were repeated three times. The experiments were complete randomized designs and were analyzed accordingly. The data were normalized with a log transformation.

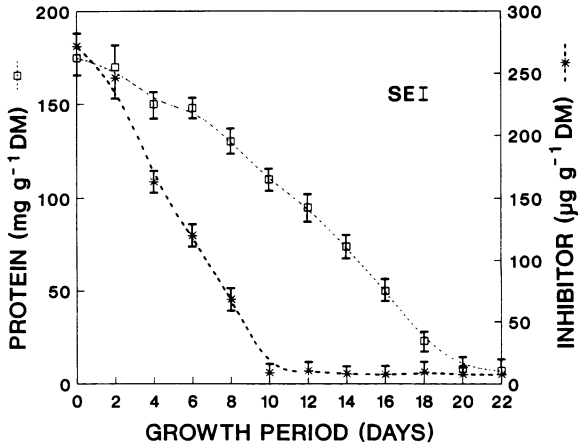

Figure 1. The t-PA inhibitor and protein content of cotyledons of *E. caffra* seeds during seedling growth.

RESULTS

The t-PA Inhibitor Content of Developing Seeds

The t-PA inhibitor content of the seeds was low in seeds with a length of up to 6 mm. After this stage of seed development, up to the time when the seeds reached maturity, the t-PA inhibitor content of the seeds increased exponentially. It finally reached a level that was 38 times higher than the level at the early stages of seed development (Fig. 1). As with the t-PA inhibitor, the protein content of the cotyledons increased slowly from day 2 to day 6 of seed development. Thereafter, the total protein content of the cotyledons increased exponentially but at a much slower rate than the t-PA inhibitor. At maturity, the concentration of the total protein in the cotyledons was 15 times higher than the concentration during the early stages of seed development (Fig. 1). The ratio of t-PA inhibitor $\times 10^3$ to total protein over the period of seed development was constant at 0.77 from day 3 to day 6. However, from day 7 to day 10 the ratio increased from 3.2 to 14.5 and finally reached a value of 22.2. This indicates that although the total protein and the t-PA inhibitor accumulate over the same period of time, they accumulate at substantially different rates.

The t-PA Inhibitor Content of Seeds during Germination

The t-PA inhibitor content of the cotyledons decreased linearly from the onset of imbibition and reached a minimum value at the 10th day of seedling growth. At this stage, the t-PA inhibitor content of the cotyledons was 4.1% of the initial inhibitor concentration in the cotyledons before imbibition. Thereafter, the inhibitor content was maintained at this level until the cotyledons were shed from the seedlings at day 22 (Fig. 2, 3). The initial rate of disappearance of the protein was relatively slow. The protein levels decreased at a faster rate and nearly linearly from the sixth day since the onset of imbibition until day 20, when it reached a minimum concentration of 3.6% of the initial value (Fig. 2, 3). It is clear that the protein in the cotyledons decreased at a much slower rate and over a longer period of time than the t-PA inhibitor.

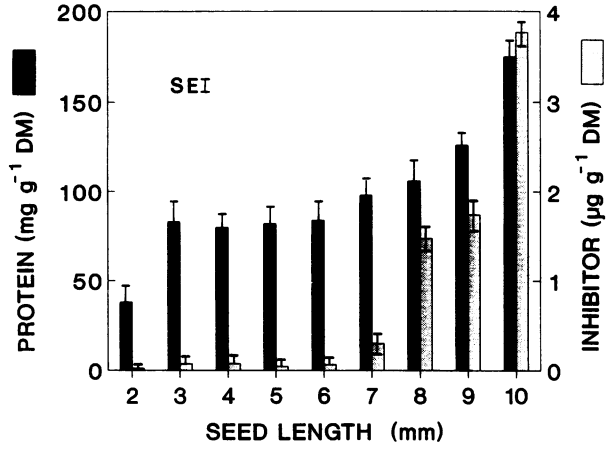

Figure 2. The t-PA inhibitor and protein content of cotyledons of *E. caffra* seeds during seed development.

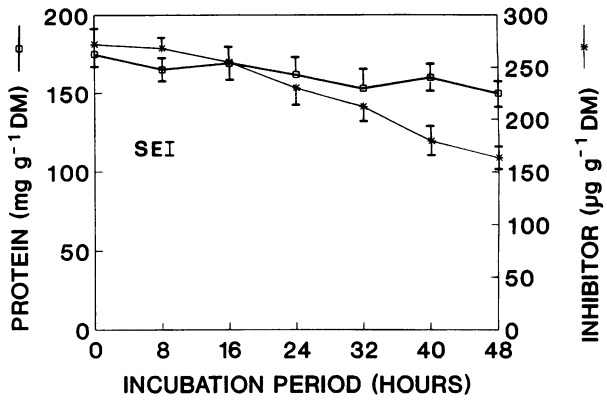

Figure 3. The effect of imbibition and germination on the t-PA inhibitor and protein content of cotyledons of *E. caffra* seeds.

The t-PA Inhibitor Content of Callus Cultures

The t-PA inhibitor contents of the calli from different organs were not different from each other (Table I). The t-PA inhibitor was present in very small amounts in callus tissue. Large differences in the t-PA inhibitor content of tissues from different organs and callus from these organs were detected. Compared with the concentration of t-PA inhibitor in plant tissue, the t-PA inhibitor content of the calli was 5 to 10 times lower than the concentration in roots, shoots, and leaves. The t-PA inhibitor content of cotyledonary callus was 700 times lower than the t-PA inhibitor content of cotyledonary tissue.

The t-PA Inhibitor Levels during the Growth Stages of the Suspension Culture

Differences were found in the t-PA inhibitor content of the different growth phases of the suspension cultures. The t-PA inhibitor level of the cell suspension increased from the lag phase through the exponential phase to the end of the linear growth phase. It then decreased in the stationary growth phase of the suspension culture (Fig. 4). The t-PA inhibitor content of the cell suspension in the linear growth phase was significantly different from the inhibitor levels in the lag and stationary growth phases.

Effect of Protein-Inducing Compounds on the t-PA Inhibitor Content of Cell Suspension Cultures

The polyamines tested—spermine and spermidine—as well as the organic acids tested—phytic, salicylic, and benzoic

Table I. *The t-PA Inhibitor Content of Callus and Tissues of E. caffra (mean ± SE)*

Tissue	t-PA Inhibitor	
	Callus	Plant tissue
	µg g^{-1} protein	
Cotyledon	3.8 ± 0.62	2662.3 ± 292.8
Leaf	3.3 ± 0.54	43.0 ± 6.4
Root	4.6 ± 0.68	23.6 ± 3.0
Shoot	5.1 ± 0.94	32.1 ± 4.1

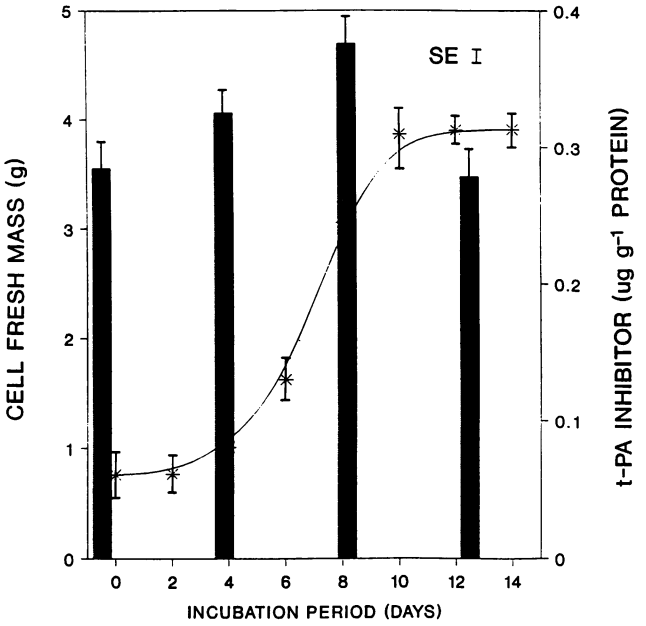

Figure 4. The t-PA inhibitor content of shoot cell suspension cultures of *E. caffra* during the different growth phases of the culture.

acid—decreased the t-PA inhibitor content of the cells marginally. Ethylene glycol chitin and the cell wall hydrolysate of *E. caffra* and *L. esculentum* had no marked effect on the t-PA inhibitor content of the cell suspension (Table II).

Effect of Cysteine and Sulfur on the t-PA Inhibitor Content of Cell Suspension Cultures

There was no significant difference between the t-PA inhibitor content of the shoot cell suspension in the basal medium (1.73 mM SO_4^{-2}) and in nutrient media with less than 17.3 mM SO_4^{-2}. The t-PA inhibitor content of the cell suspensions was markedly higher at an S^{-2} concentration of 17.3 mM (Fig. 5). This is 10 times the S^{-2} concentration in the basal nutrient medium. The different S^{-2} treatments did not have an effect on the total protein content of the suspension cultures at concentrations higher than 0.346 mM (Fig. 5). However, at 0.173 mM of S^{-2}, the protein content of the suspension cultures was substantially lower than at the higher S^{-2} levels. Growth decreased at the lowest and the highest concentrations of 0.173 and 17.3 mM S^{-2}, respectively. The viability of the cells at these SO_4^{-2} concentrations was not different from the control value (Table III). The decrease in the fresh mass of the cell suspension culture at 10 times and 0.1 times the SO_4^{-2} concentration in the basal culture medium was probably caused by a decrease in cell growth rather than cell mortality. The addition of L-cysteine to the suspension medium that contained 0.173 to 8.65 mM S^{-2} decreased the growth (Table IV) as well as the t-PA inhibitor content of the shoot cell suspension culture (Fig. 6).

Table II. *The Effect of Protein-Inducing Compounds on the t-PA Inhibitor Content of Excised Leaves and Shoot Cell Suspension Cultures of E. caffra (mean ± SE)*

Protein-Inducing Compound	t-PA Inhibitor Content	
	Leaf tissue	Cell suspension
	μg g⁻¹ protein	
Control	67.3 ± 9.7	0.40 ± 0.051
Spermine	57.0 ± 9.0	0.38 ± 0.071
Spermidine	59.8 ± 11.6	0.42 ± 0.070
Ethylene glycol chitin	65.9 ± 8.9	0.29 ± 0.034
Phytic acid	42.4 ± 5.2	0.18 ± 0.014
Benzoic acid	51.6 ± 5.8	0.27 ± 0.022
Calicyclic acid	58.3 ± 6.1	0.30 ± 0.047
Cell wall hydrolysates of		
E. caffra	62.5 ± 10.6	0.37 ± 0.062
L. esculentum	63.2 ± 11.6	0.31 ± 0.042

DISCUSSION

During the period of imbibition and germination, the t-PA inhibitor and the total protein content of the cotyledons decreased linearly. The decrease in the t-PA inhibitor in the cotyledons during the growth of the seedlings did not follow the same pattern as the decrease in the protein content. The rate of disappearance of the t-PA inhibitor was much faster and the inhibitor reached its lowest concentration in the cotyledons in half the time that the protein reached its minimum level. The t-PA inhibitor reached its minimum value approximately halfway through the lifetime of the cotyledons. At this stage, the cotyledons were still green and morphologically not different from the cotyledons at the time of germination. The onset of chlorosis in the cotyledons on day 14 could not be correlated with the pattern of t-PA disappearance in the cotyledonary tissue. The marked difference in the pattern of disappearance of inhibitor and storage protein from the cotyledons suggests that the inhibitor does not have the same function as the storage protein during the process of germination and seedling development. The t-PA inhibitor as well as trypsin inhibitors in legumes are accumulated mainly

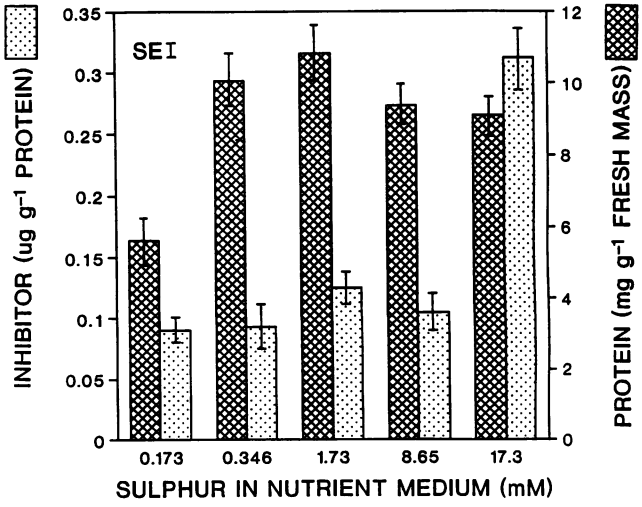

Figure 5. The effect of Na_2SO_4 on the t-PA inhibitor and protein content of shoot cell suspension cultures of *E. caffra*.

Table III. *The Effect of Na_2SO_4 on the Viability and Growth Efficiency of Shoot Cell Suspension Cultures of E. caffra after 14 d of Culture (mean ± SE)*

Na_2SO_4	Growth Index	Viability of Cells
mM		%
0.173	1.7 ± 0.9	74 ± 23
0.346	3.1 ± 1.4	70 ± 25
1.73	3.2 ± 1.7	68 ± 18
8.56	3.4 ± 1.7	77 ± 19
17.3	1.6 ± 1.0	62 ± 21

in the seeds. As with the t-PA inhibitor, most reports indicate that the trypsin inhibitor content of the seeds declines (6, 30) or fluctuates and then declines during germination and subsequent seedling growth (19).

The decrease in the t-PA inhibitor and total protein content of the cotyledons during germination suggests that the inhibitor is degraded with the increase of proteinases that catalyze the breakdown of storage protein. Some workers are of the opinion that the catalytic activity of proteolytic enzymes in the seeds is inactivated by trypsin and other proteinase inhibitors. Storage proteins are mobilized only during germination, when the proteinases are activated as the inhibitors are degraded. This could be the case in seeds of black-eyed peas (9), in which trypsin inhibitors have actually been shown to inhibit seed proteases to a certain extent. In contrast with this view, it has been reported that no endogenous soybean proteinases have been inhibited by soybean trypsin inhibitor (3) and no causal relationship existed between the increase in mung bean endopeptidase activity and the decrease in the inhibitory activity of trypsin inhibitors during germination (6). The disappearance of t-PA inhibitor during germination suggests that the breakdown products were probably utilized by the growing embryo and seedling. Sulfur-containing amino acids are underrepresented in the main storage proteins of legume seeds (11). Trypsin and chymotrypsin inhibitors, as well as the t-PA inhibitors, have a relatively high cysteine content (15, 16, 25), and can, therefore, serve as a source of sulfur-containing amino acids for the developing embryo. However, recent reports indicated that proteinase inhibitors in cotyledonary tissue can undergo specific degradation, different from the nonspecific degradation of the true reserve proteins (10, 28). The occurrence of these inhibitors and iso-inhibitors in seeds, as well as the appearance and disappearance of these inhibitors and iso-inhibitors at different times during germination (19, 23), suggests that the function of trypsin inhibitors during germination is more complex than merely being a source of nitrogen or sulfur to the seedling. However, it is not clear whether the new bands of trypsin inhibitors that appear during germination are a result of *de novo* protein synthesis or are merely a modification of the original inhibitors.

The increase in t-PA inhibitor during seed development occurred over the same period of time than the accumulation of storage protein. However, the rate of the accumulation of inhibitor was substantially faster than the rate of protein accumulation. This suggests that the inhibitor does not behave like a storage protein (22). Considering that t-PA and trypsin inhibitors accumulate in seeds (20, 22), it would be expected to accumulate along with the other storage proteins in the protein bodies. Immunocytochemical analysis indicated that soybean trypsin inhibitor was associated with the protein bodies as well as the cytoplasm, nuclei, and cell walls of soybean cotyledons (12). The association of trypsin inhibitors with the cytoplasm rather than with the protein bodies in cotyledonary tissue was reported for mung bean (2). It was suggested that the widespread distribution of trypsin inhibitors in the cytoplasm indicated that they are not related in function to true storage proteins in the cotyledons but have a protective role by preventing the proteolytic degradation of organelles and cytosolic proteins (6). Therefore, it is possible that trypsin inhibitors do not have a role in the general metabolism of vegetative tissue and probably have a function in the resting or germinating seed. The function of trypsin inhibitors in plants remains to be demonstrated. The function of trypsin inhibitors in seeds seems to be related to a nonessential metabolic process during germination or as a defense against

Table IV. *The Effect of Na_2SO_4 and L-Cysteine on the Growth and Viability of Shoot Cell Suspension Cultures of E. caffra after 14 d of Culture (mean ± SE)*

Na_2SO_4	L-Cysteine	Growth Index	Viability of Cells
mM			%
0.173	0.0	1.8 ± 1.2	72 ± 32
0.173	0.1	1.2 ± 0.8	37 ± 17
1.73	0.0	3.6 ± 1.8	68 ± 26
1.73	0.1	1.4 ± 0.7	31 ± 14
8.56	0.0	3.9 ± 1.1	70 ± 17
8.56	0.1	1.2 ± 0.8	29 ± 11

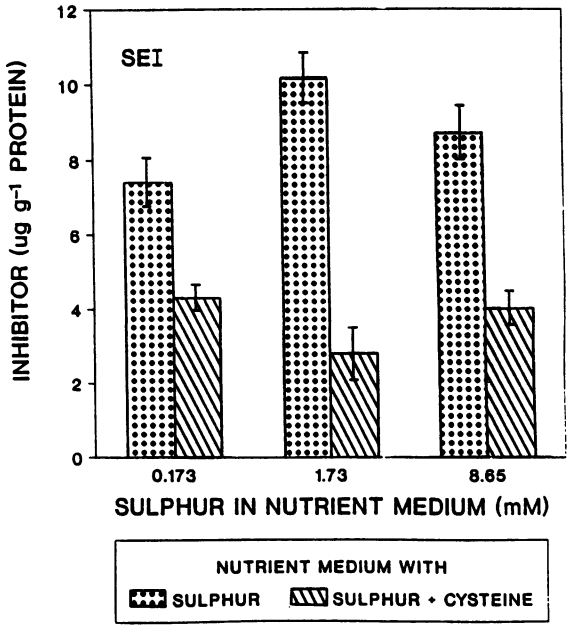

Figure 6. The effect of L-cysteine and Na_2SO_4 on the t-PA inhibitor and protein content of shoot cell suspension cultures of *E. caffra*.

insect predators (1). The role of trypsin inhibitors in plants has been questioned since it was reported that soybean seeds lacking trypsin inhibitors germinated and the resulting plants completed a normal life cycle (13). However, inhibitors of blood plasma-related enzymes such as t-PA and plasmin inhibitors (21) in seeds and their evolutionary development remain obscure. The observation that the reactive site for the inhibition of t-PA is different from that for trypsin inhibition (15) excludes the coincidental inhibition of t-PA by the same reactive site as for trypsin inhibition. An interesting possibility as to why trypsin inhibitors in soybean, and probably other plants, have been maintained during breeding and selection may be the linkage of an acid phosphatase locus to the trypsin inhibitor (13).

Callus derived from root, shoot, leaf, and cotyledonary tissue grew well on the basal medium supplemented with 10 μM BA and 5 μM 2,4-D. Shoot callus grew faster on this medium than the callus derived from other tissues. Leaf callus became brown after long periods of culture, while callus from other tissues maintained a white to creamy color. Cotyledonary callus was hard and root callus grew at a very slow rate. Shoot callus was considered as best for the establishment of a suspension culture because it grew well, did not become brown, and friable lines were easily selected. The accumulation of t-PA inhibitor does not seem to be affected by cell growth activity, as was indicated by the effect of S^{-2} levels in the nutrient medium. However, the t-PA inhibitor seems to be present in the cells in the exponential and linear growth phases of the suspension culture of *E. caffra*. Differences were observed in the protein and protease inhibitor levels during the different growth stages of the shoot cell suspension cultures of members of the Solanaceae such as *Scopolia japonica,* tobacco, and tomato. The levels of proteinase inhibitors was reported to increase markedly with an increase in the age of the culture (18, 27, 29).

The inhibitor of t-PA does not accumulate in large amounts in callus or suspension cultures. In contrast with these results, chymotrypsin inhibitors were reported to accumulate in tobacco (*Nicotiana tabacum* L.) callus cultures (29) and in tomato (*L. esculentum* var VFNT Cherry X *L. peruvianum*) suspension cultures (27). A trypsin inhibitor was observed to accumulate in carrot suspension cultures (5). A plasmin inhibitor was reported to accumulate at higher levels in cell suspensions than in the intact plant tissue of *S. japonica* (18).

The levels of proteinase inhibitors can be increased *in vitro* with the addition of specific chemicals to the suspension culture medium. Suspension cultures of lucern, tomato, and tobacco were found to accumulate proteinase inhibitors when ethylene glycol chitin, chitosan, or a proteinase inhibitor inducing factor from hydrolyzed cell walls of tomato or the mother plant was added to the suspension medium (27). The addition of hydrolyzed cell wall extracts from tomato and *E. caffra* to shoot cell suspension cultures of *E. caffra* did not increase the t-PA inhibitor content of leaf tissue or cell suspensions. All plants do not react like tomato and potato on the tomato proteinase inhibitor inducing factor or endogenous inducers of proteinase inhibitor (26). This is also true for the t-PA inhibitor of *E. caffra*. Various compounds were reported to increase pathogenesis-related proteins in tobacco (24). However, the organic acids used, such as salicylic, benzoic, and phytic acid, did not increase the t-PA inhibitor content of *E. caffra* leaf tissue or shoot cell suspension cultures. These compounds seem to be specific in inducing the accumulation of pathogenesis-related proteins.

The regulatory effect of sulfur on the synthesis of sulfur-rich proteins has not yet been reported for *in vitro* cultures. However, it has been demonstrated with leguminous seeds. Low levels of S^{-2} in lupines resulted in a decrease in the sulfur-rich conglobulin levels of the seeds without affecting the levels of the conglobulin fraction with a low cysteine content (4). The t-PA inhibitor and the other trypsin and chymotrypsin inhibitors in *E. caffra* are proteins with a relatively high S^{-2} content in the form of cysteine residues (15). In contrast to inorganic sulfur-containing compounds, the addition of a sulfur-containing amino acid such as cysteine to the suspension culture medium resulted in a decrease in the t-PA inhibitor content, viability, and growth of the suspension cultures of *E. caffra*. Addition of sulfur-containing amino acids to suspension cultures has been reported to be detrimental to the growth of suspension cultures of rice (8). The results with the cell suspension culture of *E. caffra* clearly indicate that t-PA inhibitor can be increased with SO_4^{-2} in the nutrient medium, but not by the addition of cysteine.

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